The protective mechanism of action of amines in diphtheria toxin treated Vero cells[1]

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Accepted February 23, 1982

DORLAND, R. B. 1982. The protective mechanism of action of amines in diphtheria toxin treated Vero cells. Can. J. Microbiol. **28**: 611–617.

The mechanisms by which a number of alkylamines and ethylenediamine derivatives protect Vero cells from diphtheria toxin were studied. The protective alkylamines and ethylenediamine compounds blocked the cellular degradation of diphtheria toxin, but did not prevent bulk toxin uptake. Specific antibody neutralized the inhibitory effects of toxin on protein synthesis in amine-treated cells when added at 37°C, but was ineffective when added at 4°C. Analogous results were obtained when inositol hexaphosphate was used in the place of specific antibody. Both experiments suggested that, in the presence of the amine compounds studied, diphtheria toxin is sequestered intracellularly and is recycled to the surface for antibody or inositol hexaphosphate neutralization. Finally, it was shown that the protective amines markedly increased the intralysosomal pH of Vero cells and that when the pH of the culture medium was lowered to 4.5, the amine-mediated protective effect was bypassed. In general, the results suggest that the amines exert their protective effect at the level of some acidic intracellular vesicle population, possibly the lysosomes, and thus may indicate a crucial role for the lysosomes in the generation of cytotoxicity.

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On a étudié les mécanismes par lesquels un certain nombre d'alkylamines et de dérivés de l'éthylènediamine ont un effet protecteur sur des cellules Vero contre la toxine diphtérique. Ces alkylamines et ces composés de l'éthylènediamine protecteurs bloquent la dégradation cellulaire de la toxine diphtérique, mais n'empêchent pas la pénétration de la toxine brute. Chez des cellules traitées aux amines, les effets inhibiteurs de la toxine sur la synthèse protéique peuvent être neutralisés par un antisérum spécifique si la réaction se fait à 37°C, mais ce sérum est inefficace à 4°C. Des résultats similaires sont obtenus en utilisant de l'inositol héxaphosphate au lieu de l'antisérum spécifique. Ces deux expériences laissent croire qu'en présence des amines étudiées la toxine diphtérique serait sequestrée à l'intérieur de la cellule et retournée à la surface, ce qui explique la neutralisation par l'anticorps ou l'inositol héxaphosphate. Finalement, il a été démontré que ces amines protectrices augmentent considérablement le pH intralysosomal des cellules Vero et que, lorsque le pH du milieu de culture est abaissé à 4,5, l'effet protecteur lié aux amines est annulé. Globalement, les résultats suggèrent que les amines excercent leur effet protecteur au niveau de vésicules intracellulaires acides, possiblement les lysosomes, ce qui indiquerait que ces lysosomes ont un rôle crucial dans le déclenchement de la cytotoxicité.

[Traduit par le journal]

Introduction

The cytotoxic effect of diphtheria toxin in eucaryotic systems results from the toxin-catalyzed ADP-ribosylation of a macromolecule essential for protein synthesis, elongation factor 2 (EF-2) (Collier 1975). The ADP-ribosylated EF-2 is inactive; protein synthesis thus ceases and the toxin-treated cell dies. A number of steps must occur, however, before the enzymatically active A fragment of diphtheria toxin can enter the cytoplasm to initiate the ADP-ribosylation reaction. First, the toxin molecule binds to specific receptors on the plasma membrane of sensitive cells (Middlebrook et al. 1978). After binding, toxin or active fragment is translocated in some manner to the cytoplasm. One possibility is that toxin simply diffuses across the plasma membrane by virtue of a hydrophobic sequence in the B region (Pappenheimer 1977); other evidence indicates that the toxin is internalized by receptor-mediated endocytosis and subsequently delivered to the lysosomes (Dorland et al. 1979). The fact that chloroquine, a potent lysosomotropic agent, simultaneously blocks the degradation of diphtheria toxin and prevents toxin-mediated cytotoxicity implies that the lysosome plays an important role in the generation of active fragment (Leppla et al. 1980). Results obtained by Draper and Simon (1980) and Sandvig and Olsnes (1980), suggesting that diphtheria toxin sequestered in endocytic vesicles is translocated to the cytoplasm in response to an acid pH environment, similarly imply lysosomal involvement in the intoxication pathway.

It has been shown previously that alkylamines protect sensitive cells from the actions of diphtheria toxin or *Pseudomonas* exotoxin A (Leppla et al. 1982[2]), though the mechanism(s) of the protective effect was unclear.

[1]The views expressed in this article are of the author and do not purport to reflect the positions of the Department of the Army or the Department of Defense.

[2]S. H. Leppla, R. B. Dorland, J. L. Middlebrook, and J. D. White. 1982. Interaction of *Pseudomonas* endotoxin A with sensitive mammalian cells. Rev. Infect. Dis. In press.

The data presented here demonstrate that a large number of alkylamines simultaneously protect Vero cells from diphtheria toxin and block toxin degradation, as determined by measuring excretion of trichloroacetic acid (TCA) soluble radioactivity into the culture medium. Concomitant spectrofluorimetric experiments strongly suggest that the site of action of these drugs is the lysosome and further support the view that lysosomal activity is required for release of toxic fragment A to the cytoplasm.

Materials and methods

Cells and cell culture

Seed stock for the Vero cell line was obtained from the American Type Culture Collection (ATCC). The lines were maintained in 700-cm^2 roller bottles (Costar No. 1234) with the medium and serum supplement recommended by ATCC.

Media and sera

All media, vitamins, antibiotics, and amino acids were obtained from Grand Island Biological Co., Grand Island, NY. Fetal calf serum (FCS) was purchased from Reheis, Phoenix, AZ, and heat inactivated for 30 min at 56°C before use in cell culture experiments.

Materials

"Low pH" carrier-free sodium iodide-125 and L-[3,4, 5-^{3}H(N)]leucine (135 Ci/mmol, 1 Ci = 37 GBq) were obtained from New England Nuclear, Boston, MA. Inositol hexaphosphate (IHP) was obtained from P-L Biochemicals, Inc., Milwaukee, WI. Deoxyribonuclease I (DNAse I), type I, fluorescein isothiocyanate labeled dextran (FITC–dextran) (average molecular weight 70 000), and chloroquine were purchased from Sigma Chemical Co., St. Louis, MO. Ethylenediamine (ED) and the substituted ED derivatives, tetramethyl-ED, tetraethyl-ED, *N,N*-dimethyl-ED, and *N,N'*-dimethyl-ED, were purchased from Aldrich Chemical Co., Inc., Metuchen, NJ. Methylamine, ethylamine, *n*-propylamine, *n*-butylamine, triethylamine, and tributylamine were purchased from Eastman Kodak Co., Rochester, NY. All other chemicals were reagent grade and were used without further purification.

Toxin

Diphtheria toxin was obtained from Connaught Laboratories, Toronto, Ont., Canada, and purified by chromatography over DEAE-cellulose (DE-52). The final product ran as a single band on sodium dodecyl sulfate – polyacrylamide gel electrophoresis. The toxin was radiolabeled as previously described (Middlebrook et al. 1978) using chloramine T by the method of Roth (1975) to a specific activity of 1×10^7 to 2×10^7 cpm/μg (0.1–0.2 mol iodine/mol toxin). The radiolabel was approximately equally distributed between fragments A and B. The labeling procedure had no detectable effect on the biological activity of the toxin as determined by cytotoxicity assay. Diphtheria toxin specific antiserum, obtained by hyperimmunization of a goat, was prepared using purified diphtheria toxoid donated by Dr. R. Holmes, Uniformed Services University of Health Sciences.

Inhibition of protein synthesis assay

Cells in 24-well tissue culture plates were equilibrated at 37°C in leucine-free Hanks' 199 medium (H199) supplemented with 5% FCS and 25 m*M* *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid (Hepes) buffer, pH 7.4 (complete H199). The cells were preincubated for 30 min with drug and then toxin was added to the desired concentration and incubation continued for 2 h. At this point, L-[^{3}H]leucine was added to a final concentration of 0.2 μCi/mL and the amount of protein synthesis was determined following a further 1.5-h incubation period. The monolayers were rinsed once with Hanks' balanced salts solution (HBSS) containing 1 mg/mL L-leucine, once with unsupplemented HBSS, and lysed by addition of 100 μL of a 1:1 mixture, made immediately before use, of 0.2% sodium dodecyl sulfate dissolved in water and 0.5 mg/mL DNAse I dissolved in HBSS containing 0.05 *M* Hepes, 2 m*M* $MgCl_2$, and 2 m*M* $CaCl_2$, pH 7.5. The plates were shaken for 5 min or until cell lysis was complete as determined by light microscopy. Numbered 11-mm diameter paper discs (Schleicher and Schuell, No. 740-E) were dropped into the wells to absorb the cell lysates. After 1–2 min, 1.0 mL of cold 10% TCA was added to each well. The discs were collected in a bottle and washed sequentially with 5% TCA, ethanol – ether (1:1), and ether. The discs were air-dried and counted in Liquiflour–toluene scintillation solution (New England Nuclear) in a Searle Analytic Mark III liquid scintillation counting system.

Binding and degradation assays

Details of the binding (Middlebrook et al. 1978) and degradation (Dorland et al. 1979) assays have been described.

Fluorimetric determination of intralysosomal pH

Cells growing in 75-cm^2 T-flasks were incubated for 18 h at 37°C with FITC–dextran (1 mg/mL). The cells were then rinsed four times with HBSS, trypsinized, rinsed once in complete Earles' 199 (E199) and twice with HBSS, and resuspended in HBSS containing 2% FCS and 25 m*M* Hepes buffer. Fluorescence intensity in the absence or presence of amines was determined using a Perkin-Elmer MPF-4 fluorescence spectrophotometer.

Results

Effect of amines on diphtheria toxin induced inhibition of protein synthesis and toxin degradation

The ability of the amine compounds to protect Vero cells from diphtheria toxin as determined by inhibition of protein synthesis assay is shown in Table 1. Previous studies (Kim and Groman 1965; Ivins et al. 1975; Bonventre et al. 1975; Middlebrook and Dorland 1977) have shown that NH_4Cl effectively protects cells from the action of diphtheria toxin. Those findings were confirmed here. Similarly, the alkylamines, methylamine, ethylamine, *n*-propylamine, *n*-butylamine, triethylamine, and tributylamine, prevented the toxin-induced inhibition of protein synthesis, as did the substituted ED derivatives. ED itself was ineffective. Parallel results were obtained using a 3-h cytotoxicity

TABLE 1. Effect of amines on diphtheria toxin degradation and toxin-induced inhibition of protein synthesis

	% control degradation	% inhibition of protein synthesis
Control	100	93
NH_4Cl	114	7
Methylamine	73	27
Ethylamine	20	ND
Propylamine	26	3
Butylamine	11	10
Triethylamine	8	23
Tributylamine	36	22
ED	98	91
Tetramethyl-ED	6	26
Tetraethyl-ED	3	0
N,N-dimethyl-ED	4	0
N,N'-dimethyl-ED	7	0

NOTE: To assay for diphtheria degradation, cells were incubated overnight at 4°C with 0.03 μg/mL ^{125}I-labeled toxin. The cells then were rinsed three times with HBSS, fresh complete medium containing the indicated amines (10 m*M* concentrations) was added, and the cells were warmed to 37°C. After 3 h, triplicate samples were assayed for TCA-soluble radioactivity as described in Materials and methods. To assay for toxin-induced inhibition of protein synthesis, cells were preincubated for 30 min at 37°C with the indicated amines (10 m*M*). Diphtheria toxin (10 ng/mL) was added and incubation was continued for 2 h. The cells then were pulsed with [^{3}H]leucine (1 μCi/mL) and incorporation of radioactivity into TCA-precipitable form was determined in triplicate samples as described in Materials and methods. ND, not done.

assay (data not shown). Concomitant experiments demonstrated that, with the exception of NH_4Cl, those compounds that blocked the toxin-induced inhibition of protein synthesis also blocked toxin degradation. NH_4Cl, though fully protective, had no detectable effect on degradation, as previously described (Dorland et al. 1981). Methylamine partially inhibited degradation, while the other alkylamines tested had substantial (80–90%) inhibitory activity. ED had no effect on toxin degradation, but the ED derivatives were potent inhibitors. None of the tested compounds had detectable effect on internalization of surface-bound toxin in cells prebound at 4°C (data not shown), as measured by the pronase–IHP technique (Dorland et al. 1979).

Effect of amines on diphtheria toxin uptake

As previously shown, the uptake of radiolabeled diphtheria toxin by sensitive cells at physiological temperature follows a biphasic pattern (Middlebrook et al. 1978), increasing to a peak at 1–1.5 h and subsequently decreasing. Further studies (Middlebrook and Dorland 1981) suggested that this pattern results from both (1) toxin-mediated depletion of available cell surface receptors via endocytosis and lysosomal degradation and (2) toxin-induced inhibition of protein synthesis, which markedly reduces toxin–receptor binding capacity. In the presence of NH_4Cl, toxin uptake does not follow this biphasic pattern, but reaches a plateau approximately fourfold greater than the control peak level (Dorland et al. 1981). Comparable results were obtained with the other protective amines (Table 2). At 5 h, radioactivity associated with the control cells had decreased to 500 cpm (peak cell-associated radioactivity = 2400 cpm). In the presence of the protective amines, however, cell-associated radioactivity at 5 h remained high. ED, which had no protective effect (Table 1), did not influence the biphasic uptake pattern; cell-associated radioactivity in the ED-treated cells was equivalent to that in the control cells.

Neutralization of diphtheria toxin by specific antibody in the presence of amines

Previous studies have shown that NH_4Cl appears to maintain diphtheria toxin in a position accessible to antibody neutralization (Ivins et al. 1975; Bonventre et al. 1975), possibly at the cell surface or within an intracellular vesicular pool that equilibrates rapidly with the cell surface. In similar studies using the alkylamines (Table 3) the cells were exposed to diphtheria toxin in the presence or absence of amines for 60 min at 37°C. At this point, either toxin-specific antibody was added and incubation continued at 37°C or the cells were rapidly chilled to 4°C, toxin-specific antibody was added, and incubation was continued at 4°C. The cells were then rinsed thoroughly, fresh complete medium was added, and protein synthesis was assayed 24 h later.

The results in Table 3 show that, under these

TABLE 2. Effect of amines on uptake of diphtheria toxin by Vero cells

	Cell-associated radioactivity, cpm ^{125}I		
	1 h	3 h	5 h
Control	2400	800	500
NH_4Cl	2200	5500	6100
Methylamine	2300	5800	7800
Ethylamine	2200	3100	3700
Propylamine	2500	3900	3900
Butylamine	2600	2900	4400
Triethylamine	2400	7000	8000
Tributylamine	2200	3300	3000
ED	2900	800	600
Tetramethyl-ED	3000	7000	9400
Tetraethyl-Ed	2800	5800	7300
N,N-dimethyl-ED	3100	5400	6200
N,N'-dimethyl-ED	3200	6500	9100

NOTE: Cells were preincubated for 30 min at 37°C with the indicated amines (10 m*M*). ^{125}I-labeled diphtheria toxin (0.03 μg/mL) was then added and incubation was continued at 37°C. At the times indicated, triplicate samples were rinsed three times with HBSS, solubilized in 0.1 *M* NaOH, and cell-associated radioactivity was determined as described in Materials and methods.

TABLE 3. Protection of Vero cells from diphtheria toxin by amines: effect of antitoxin addition

	% control protein synthesis		
	No antitoxin addition	+ antitoxin at 37°C	+ antitoxin at 4°C
Control	0	3	4
NH_4Cl	2	85	17
Methylamine	3	60	30
Ethylamine	1	87	29
Propylamine	1	82	24
Butylamine	1	87	11
Triethylamine	1	63	27
Tributylamine	4	89	21
ED	0	7	5
Tetramethyl-ED	7	75	48
Tetraethyl-ED	10	71	40
N,N-dimethyl-ED	27	57	39
N,N'-dimethyl-ED	30	63	54
Chloroquine	2	60	30

NOTE: Cells were preincubated with the indicated amines (10 m*M*) or chloroquine (0.1 m*M*) for 15 min at 37°C. Diphtheria toxin (3 ng/mL was then added and incubation was continued at 37°C for 60 min. At this point, antitoxin (1:500) was added for 30 min at 37°C (column 2) or 4°C (column 3). No antitoxin was added to the control cells (column 1). The cells then were rinsed, fresh complete medium was added, and incubation was continued for 24 h at 37°C. Protein synthesis was then assayed as described in Materials and methods.

TABLE 4. Protection of Vero cells from diphtheria toxin by amines: effect of IHP addition

	% control protein synthesis		
	No IHP addition	+ IHP at 37°C	+ IHP at 4°C
Control 1	>1	3	3
NH_4Cl	8	59	14
Methylamine	14	65	21
Ethylamine	13	81	19
Propylamine	13	72	17
Butylamine	13	66	10
Triethylamine	9	59	11
ED	>1	3	4
Tetramethyl-ED	20	67	15
Tetraethyl-ED	21	56	25
N,N-dimethyl-ED	21	53	21
Control 2	63	65	69

NOTE: Cells were preincubated with the indicated amines (10 m*M*) for 15 min at 37°C. Diphtheria toxin (1 ng/mL) was then added and incubation was continued at 37°C for 60 min. The control 2 cells received IHP at a final concentration of 10 mg/mL simultaneously with toxin (columns 1, 2, and 3). Following the 60-min incubation period, IHP (10 mg/mL) was added for 30 min at 37°C (column 2) or 4°C (column 3). No IHP was added to the cells in column 1. The cells were then rinsed, fresh complete medium was added, and incubation was continued for 24 h at 37°C. Protein synthesis was then assayed as described in Materials and methods.

conditions, essentially no amine-mediated protection was detectable in the absence of added antibody (column 1). When antibody was added to cells maintained at 37°C, substantial protection was obtained in the presence of all the compounds tested, with the exception of ED (column 2). The observed protective effect was considerably reduced when antibody was added to cells maintained at 4°C (column 3). Antibody was ineffective when added at 60 min to toxin-treated control cells at either 37 or 4°C; however, complete protection was obtained when antibody was added simultaneously with the toxin at zero time.

Effect of IHP on diphtheria toxin treated cells in the presence of amines

The data presented in Table 4 suggest that at 37°C, in the presence of amines, diphtheria toxin is available at the cell surface. IHP has been shown previously to elicit dissociation of surface-bound diphtheria toxin (Dorland et al. 1979). In the experiments presented here, cells were preincubated in the presence or absence of selected amine compounds. IHP was then added either at 37 or 4°C, following which the cells were rinsed, fresh complete medium was added, and protein synthesis was assayed 24 h later as described in Materials and methods.

Results (Table 4) show that cells treated with IHP at 37°C (column 1) were more effectively protected from diphtheria toxin than were cells treated with IHP at 4°C (column 2). Protection, however, did not reach the levels attained with toxin-specific antibody. In the control samples, IHP treatment provided 60–70% protection from diphtheria toxin (Table 5, control 2) as opposed to 95–100% protection provided by specific antibody. No protection was observed in the absence of IHP treatment.

Fluorimetric measurement of intralysosomal pH in the presence of amines

Since the protective alkylamines and ED derivatives all markedly blocked the degradation of radiolabeled toxin, it appeared reasonable to hypothesize that these compounds act at the level of the lysosome. A modification of the fluorimetric technique of Okhuma and Poole (1978) therefore was used to determine whether these compounds were concentrated in lysosomes. Vero cells were allowed to endocytose FITC–dextran (1 mg/mL) for 18 h. The cells were rinsed thoroughly, trypsinized, and fluorescence spectra of the final cell suspensions were measured under various conditions. Results are shown in Table 5.

Here, the amine-mediated increase in relative fluorescence intensity presumably reflects an amine-mediated increase in intralysosomal pH. Since the lysosomal proteases have pH optima in the range of 4.5–4.8, enzyme inhibition at increased pH is thought to be a

TABLE 5. Effect of amines on intralysosomal pH in Vero cells

	Intralysosomal pH
Control	4.6±0.3
NH_4Cl, 10 m*M*	6.7±0.8
Methylamine, 10 m*M*	6.5±0.7
Ethylamine, 10 m*M*	6.5±0.9
Propylamine, 10 m*M*	6.2±0.4
Butylamine, 10 m*M*	6.1±0.3
Triethylamine, 10 m*M*	5.9±0.3
Tributylamine, 10 m*M*	5.7±0
ED, 10 m*M*	4.4±1.0
Tetramethyl-ED, 10 m*M*	6.3±0.5
Chloroquine, 0.1 m*M*	6.3±0

NOTE: Cells were incubated 18 h at 37°C in complete E199 medium containing FITC–dextran (1 mg/mL). The cells were then rinsed four times with HBSS, trypsinized, and resuspended in complete E199 medium. These cells were then rinsed twice in unsupplemented HBSS by centrifugation at 1000 rpm for 5 min and finally resuspended in warm HBSS supplemented with 25 m*M* Hepes buffer and 2% FCS. Fluorescence spectra of 2-mL aliquots of the final cell suspensions in the presence or absence of the indicated amines were measured in the range of 400–500 nm using a Perkin-Elmer MPF-4 fluorescence spectrophotometer. The ratio of fluorescence measured with excitation at 495 nm to that with excitation at 450 nm (Ex 495/Ex 450) was determined for each sample. These data were used to estimate intralysosomal pH based on comparisons with a standard curve generated from the fluorescence spectra of stock solutions of FITC–dextran adjusted to various pHs in the range 2.0–9.0. Results are listed as the average of three spearate experiments ± the standard error of the mean.

primary mechanism of action of the lysosomotropic amines (de Duve et al. 1974; Okhuma and Poole 1978). The data in Table 5 demonstrate that the protective amines appear to increase intralysosomal pH by approximately 2 pH units. The nonprotective compound, ED, did not appear to elicit a pH increase.

Effect of extracellular pH on the amine-mediated protection of Vero cells from diphtheria toxin

Both Draper and Simon (1980) and Sandvig and Olsnes (1980) in their studies of diphtheria toxin entry into sensitive cells and Helenius et al. (1979) in their study of BHK-21 infection by Semliki Forest virus found that an acidic extracellular pH effects the bypass of lysosomotropic amine-mediated protection. This was taken to suggest that the biologically active moieties of toxin and virus normally enter the cytoplasm from an "acidified vesicle" such as a lysosome. Similar results were obtained with the protective alkylamines in the Vero cell system (Table 6). In these experiments, cells were treated with toxin in the presence of amines, then exposed to either pH 7.4 or 4.5 in fresh complete medium, and finally assayed for protein synthesis. Results show that cells treated at physiological pH were protected from diphtheria toxin by the tested amines, with the exception of ED (column 1); cells exposed to acidic pH were no longer subject to amine-mediated protection (column 2).

TABLE 6. Effect of extracellular pH on amine-mediated protection of Vero cells from diphtheria toxin

	% control protein synthesis	
	pH 7.4	pH 4.5
Control	4	3
NH_4Cl	46	15
Methylamine	68	12
Ethylamine	88	14
Propylamine	ND	ND
Butylamine	50	9
Triethylamine	47	5
Tributylamine	60	11
ED	4	1
Tetramethyl-ED	95	20
Tetraethyl-ED	68	20
N,*N*-dimethyl-ED	79	17
N,*N*′-dimethyl-ED	88	14
Chloroquine	55	11

NOTE: Cells were preincubated for 30 min at 37°C with the indicated amines (10 m*M*) or chloroquine (0.1 m*M*). Diphtheria toxin (3 ng/mL) was then added and incubation was continued for 60 min at 37°C. The cells were then rinsed three times with HBSS, and fresh complete medium plus amines was added, adjusted to either pH 7.4 (column 1) or pH 4.5 (column 2). Incubation was continued for 30 min at 37°C . The medium then was removed, low leucine medium plus amines at pH 7.4 was added, and incubation was continued for 3 h at 37°C. Protein synthesis was then determined as described in Materials and methods. ND, not done.

Discussion

The results presented here demonstrate that both the simple alkylamines and the ED derivatives effectively protect cells from the action of diphtheria toxin (Table 1). Furthermore, the data imply that the protective effect is exerted at the level of an acidified intracellular vesicle such as the lysosome. Results from other laboratories (Maxfield et al. 1979; Davies et al. 1980) have suggested that ammonia and amines prevent the internalization of macromolecules such as α_2-macroglobulin and certain polypeptide hormones by blocking the transglutaminase-mediated clustering of ligand–receptor complexes prior to endocytosis. Subsequent studies (King et al. 1980), however, indicated that, in the presence of amines, these ligands were not maintained on the cell surface but sequestered in intracellular vesicles. Similarly, early experiments with NH_4Cl (Bonventre et al. 1975; Ivins et al. 1975) were interpreted to indicate that this compound exerted its protective effect on diphtheria toxin treated cells by preventing the internalization of surface-bound toxin molecules. More recent work (Dorland et al. 1981) has shown that internalization and degradation of diphtheria toxin appear to proceed normally in the presence of NH_4Cl, though a fraction of potentially active toxin molecules was maintained in a position accessible to antitoxin for long periods at 37°C. A series of experiments recently

published by Draper and Simon (1980) showed that NH_4Cl maintained toxin in an antibody-accessible position at 37°C, but not at 4°C. We have confirmed these results in our system using both NH_4Cl and a wide range of alkylamines (Table 3). One reasonable interpretation of these data is that, in the presence of NH_4Cl, toxin becomes sequestered in intracellular vesicles; thus, at 37°C, toxin-specific antibody is endocytosed and neutralizes intravesicular toxin, or the toxin-containing vesicles are recycled to the plasma membrane where the antibody–toxin neutralizing interaction occurs. At 4°C, presumably neither endocytosis nor exocytosis takes place; therefore, either toxin-specific antibody cannot enter the cell or intracellular toxin-containing vesicles cannot return to the cell surface.

Precedents exist for the recycling of intracellular vesicles to the plasma membrane; for example, the work of Schneider et al. (1979) with surface-bound anti-plasma membrane immunoglobulins indicates a vesicle recycling mechanism. The results shown in Table 4 provide some support for the existence of such a mechanism in the amine-treated Vero cells. IHP is a highly charged compound and as such probably does not readily cross the cellular plasma membrane barrier. It has been shown to effectively protect cultured cells from diphtheria toxin (data not shown), presumably by acting at the level of binding to the cell surface; when added to cells simultaneously with radiolabeled diphtheria toxin, IHP blocks toxin–receptor binding (Proia et al. 1979). Furthermore, when added at 4°C to cells prebound with radiolabeled diphtheria toxin, IHP elicits the detachment of the bound toxin molecules from the cell surface (Dorland et al. 1979). When IHP was substituted for antibody in temperature shift experiments (Table 4), results showed that, as in the antibody experiments (Table 3), the amine-treated cells were protected at 37°C, but not at 4°C. These results may indicate that toxin contained in intracellular vesicles is returned to the cell surface for neutralization.

Results obtained using the fluorescence probe technique of Okhuma and Poole (1978) (Table 5) demonstrate that the protective amines elevate the intralysosomal pH in Vero cells by approximately 2 pH units, presumably due to the permeation of the lysosomal membrane by the protonated form of the amine, accompanied by proton leakage out of the organelle (de Duve et al. 1974). With the exception of NH_4Cl, these amines also block the degradation of radiolabeled diphtheria toxin (Table 1). Since the lysosomal enzymes have pH optima in the range of 4–5 (Okhuma and Poole 1978), it is possible that the amine-mediated elevation of lysosomal pH leads to the inhibition of some enzyme(s) essential for the generation of active fragment. ED, which had no measurable protective effect (Table 1), did not alter lysosomal pH. It may be that ED does not enter the cell as readily as the other amine compounds tested; there is also the possiblity that ED, once internalized, it not as effectively concentrated in lysosomes. A recent study by Okhuma and Poole (1981) demonstrates that, though able to induce vacuolation in mouse peritoneal macrophages, ED is markedly less vaculogenic than ammonia and a number of tested alkylamines, implying less effective uptake.

An alternative explanation for the protective effect of intralysosomal pH elevation derives from the work of Draper and Simon (1980). Their results show that diphtheria toxin (or fragment A) rapidly penetrates the plasma membrane upon exposure to an extracellular acid pH environment. Furthermore, acidification of the extracellular environment effectively bypasses the protective effect of the lysosomotropic amines. A model was thus proposed in which diphtheria toxin sequestered in endocytic vesicles becomes exposed to acid pH, possibly following vesicle–lysosome fusion. At acid pH, diphtheria toxin or fragment A rapidly penetrates the vesicle membrane and enters the cytoplasm where it catalyzes the ADP-ribosylation of EF-2. In the presence of the protective amines, lysosomal pH is elevated such that the escape of fragment A to the cytoplasm is blocked.

In our system, results using a large number of alkylamines and ED derivatives demonstrated that the protective effect was abrogated by lowering extracellular pH to 4.5 (Table 6). Similar results were obtained with NH_4Cl and chloroquine.

These data appear to support an intracellular rather than a cell-surface mechanism of action for the protective amines. In the presence of amines, surface-bound ^{125}I-labeled diphtheria toxin is internalized normally by cells prebound with toxin at 4°C (data not shown); at 37°C, radiolabeled toxin is rapidly taken up from the medium and appears to accumulate intracellularly (Table 2). The temperature shift experiments using toxin-specific antibody (Table 3) imply that, in the presence of amines, toxin is incorporated into intracellular vesicles; results obtained with IHP (Table 4) may indicate that these vesicles are recycled to the cell surface. These data alone do not rule out the possibility that, in the presence of amines, toxin is retained at the cell surface and is simply masked by membrane components or antigenically changed at 4°C. However, in conjunction with the evidence that the alkylamines and ED derivatives (1) increase intralysosomal pH, (2) block toxin degradation, and (3) exert a protective effect that is bypassed by exogenous acid pH treatment, the results suggest an intravesicular or intralysosomal mechanism of action. Analogous results were obtained with chloroquine, another lysosomotropic agent. NH_4Cl, though showing many similarities to the other protective amine compounds, had no apparent effect on toxin degradation

(Table 1) (Dorland et al. 1981). It is possible that toxin degradation occurs in an NH_4Cl-insensitive subpopulation of intracellular vesicles and bears no relationship to the biologic (cytotoxic) effect; however, the true explanation is as yet unknown. In general, results presented here indicate an intravesicular or intralysosomal site of amine-mediated protection and suggest an essential role for the lysosomes in the generation of diphtheria toxin induced cytotoxicity.

Acknowledgements

The author thanks Drs. John Middlebrook and Stephen Leppla for many valuable discussions and Steven Knight for technical assistance.

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